AWARD NUMBER: **W81XWH-15-1-0240**

TITLE: **A Novel Therapy for Inflammatory Bowel Disease-Dual Regulatory T-Cell Programming**

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REPORT DATE: **August 2017**

TYPE OF REPORT: **Annual**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
August 2017	Annual	1 Aug 2016 - 31 Jul 2017

4. TITLE AND SUBTITLE

A Novel Therapy for Inflammatory Bowel Disease-Dual Regulatory T-Cell Programming

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0240

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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Loma Linda, CA 92350-0001

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Inflammatory bowel disease (IBD) is a group of diseases that are conditions of chronic inflammation in the gastrointestinal tract (the GUT). Current therapies for IBD have a transient effect and are associated with significant side effects due to unintended immune suppression. Hence, a therapy that can lead to a more specific and lasting control of the gut inflammation is urgently needed. This study investigates a novel therapy that aims to induce gut-homing regulatory T (Treg) cells in the peripheral lymphoid tissues. We propose that the newly generated Treg cells can specifically home to and reinstate immune tolerance in the gut, thereby providing a long-lasting control of the chronic inflammation in the gut of IBD patients. This novel therapy is a dendritic cell (DC) that is engineered to *de novo* synthesize high concentrations of both the active vitamin D metabolite [1,25$(OH)_2$D] and retinoid acid (RA). We hypothesize that such engineered DC can home to and interact with T cells in the peripheral lymphoid tissues where the DC-derived and *de novo* synthesized focally high concentrations of 1,25$(OH)_2$D and RA can promote the induction of regulatory and gut-homing molecules respectively in the same T cell that subsequently differentiates into a gut-homing Treg cell. We will test this hypothesis using an IBD animal model, i.e. experimental colitis.

15. SUBJECT TERMS Inflammatory bowel disease, calcitriol, 1,25$(OH)_2$D, dendritic cells, CYP27B1, 25-hydroxyvitamin D 1α-hydroxylase, ALDH1a2, RALDH2, regulatory T cells, Treg, gut-homing receptor.

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE	UU	26	USAMRMC
U Unclassified	U Unclassified	U Unclassified	Unclassified		19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION: Inflammatory bowel disease (IBD) is a group of diseases that are conditions of chronic inflammation in the gastrointestinal tract (the GUT)[1]. New biological therapies have revolutionized IBD treatment[2]. These new biological drugs aim to block either the function of an inflammatory mediator or the entrance of immune cells into the intestine[2]. However, such blocking is associated with numerous drawbacks. **First**, the inflammatory mediators and immune cells, which are being blocked, are also necessary for immune defense. Hence, current therapies cause unintended immune suppression, leading to severe side effects (e.g. infections and cancers)[3-9]. **Second**, the therapeutic effect via blocking of molecules and cells is transient. Consequently, frequent administration of these medications is necessary, which further increase the chance of causing immune suppression. Hence, a therapy that can lead to a more specific and lasting control of the gut inflammation is urgently need. In this regard, regulatory T (Treg) cells are subsets of T cells that can acquire the specificity for the gut and can potentially differentiate into memory cells, which can help achieve these two goals[10]. This study aims to develop a novel therapy that can induce gut-homing regulatory T (Treg) cells in the peripheral lymphoid tissues. We propose that the gut-homing Treg cells can specifically home to and provide a lasting reestablishment of immune tolerance in the gut of IBD patients, while sparing the systemic immune defense mechanisms. To achieve this goal, we propose to engineer a DC to overexpress two genes. One gene is the cytochrome P450 family 27 subfamily B member 1 (CYP27B1) that encodes 25-hydroxyvitamin D [25(OH)D] 1α-hydroxylase (hereafter 1α-hydroxylase). The 1α-hydroxylase is the enzyme that physiologically converts 25(OH)D (the major vitamin D form in the blood) into 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] (i.e. the active vitamin D metabolite). The other gene is the aldehyde dehydrogenase 1 family member A2 (ALDH1a2) that encodes retinaldehyde dehydrogenase 2 (RALDH2). The RALDH2 is the enzyme that physiologically converts retinal (a vitamin A form) into retinoic acid (RA). We reason that such engineered DC can induce the desired gut-homing Treg cells. The rationale is that DC has been shown to specifically home to the peripheral lymphoid tissues[11-13] where the engineered DC can potentially *de novo* synthesize high concentrations of 1,25$(OH)_2$D and RA. Since 1,25$(OH)_2$D has been shown to induce Treg cells[14,15] and RA has been shown to imprint gut-homing receptors on T cells[16], such engineered DC can theoretically enhance the generation of gut-homing Treg cells. Additionally, other evidence also supports the feasibility and advantage of our proposed strategy. ***First,*** the engineered DC can be potentially converted into a tolerogenic DC, and such tolerogenic status can be stable within its *in vivo* lifespan because the high 1,25$(OH)_2$D concentration can tolerize DCs[15,17-20]. Hence, the instability concern associated with adoptive cell therapies (e.g. the *ex vivo* generated Treg cells and the tolerogenic DCs)[6-8,21] can be overcome. ***Second,*** regulatory property of the induced gut-homing Treg cells may be stable because 1,25$(OH)_2$D has been shown to up-regulate the expression of Helios that is necessary for maintaining stable regulatory property in Treg cells[22-24]. Accordingly, ***we hypothesize that*** a DC engineered to overexpress both 1α-hydroxylase and RALDH2 can specifically home to and *de novo* synthesize high concentrations of both 1,25$(OH)_2$D and RA in the peripheral lymphoid tissues for an enhanced generation of gut-homing Treg cells and for the specific and lasting reestablishment of gut immune tolerance in IBD patients. To test this hypothesis, we will produce three lentiviral plasmids for engineering DCs, i.e. the lenti-CYP for overexpressing the 1α-hydroxylase, the lenti-ALDH for overexpressing the RALDH2, and the lenti-CYP-ALDH for overexpressing both the 1α-hydroxylase and the RALDH2. We will then investigate the engineered DCs for: ***1)*** the induction of gut-homing Treg cells in the peripheral lymphoid tissues; ***2)*** the migration of the gut-homing Treg cells into the gut; ***3)*** the regulatory function of the Treg cells induced by the engineered DCs; ***4)*** the suppression of established experimental colitis. We expect to deliver a therapeutic product, i.e. the DC engineered to overexpress both the 1α-hydroxylase and the RALDH2 (DC-CYP-ALDH) as a novel therapy for IBD.

2. KEYWORDS: Inflammatory bowel disease, calcitriol, 1,25$(OH)_2$D, dendritic cells, CYP27B1, 25-hydroxyvitamin D 1α-hydroxylase, ALDH1a2, RALDH2, regulatory T cells, Treg, gut-homing receptor.

3. ACCOMPLISHMENTS:

3.1 The major goals of the project: The following is the originally proposed Statement of Work with highlighted accomplishments.

Specific Aim 1 *To study phenotypes, regulatory function, gut migration, and colitis suppression capability of the T cells in the draining lymph nodes in animals that receive no treatment, wild type DCs, or DCs carrying the over-expressed 1α hydroxylase.* We have successfully engineered DCs to overexpress the 1α-hydroxylase (DC-CYP) **(Figure 2)**. During an initial pilot study, we found that the DC-CYP, when injected once intravenously as compared to subcutaneously, provided much stronger suppression of the established experimental colitis. Therefore, except for the determination of 1,25$(OH)_2$D concentration in the draining lymph nodes and the gut-migration of the newly induced Treg cells in which the different DCs were injected subcutaneously, other experiments were performed using one intravenous injection of each of the different DCs. Since subcutaneous injection is more convenient for IBD patients, we will continue to evaluate whether multiple subcutaneous injections can reach the therapeutic effect of intravenous injection.	**Timeline (Months)**	**Site 1**
Major Task 1: *to evaluate T cell phenotypes in the lymph nodes that drain the infusion sites (dLNs) in animals subcutaneously infused with the different DCs.*	1-6	Dr. Xiaolei Tang
Subtask 1: *to determine 1,25(OH)2D concentration by ELISA in the dLNs following subcutaneous infusion of the different DCs.*	1-3	
Subtask 2: *to determine regulatory and gut-homing phenotypes of the T cells in the dLNs following subcutaneous infusion of the different DCs.*	1-6	
Milestone(s) Achieved: 1. Subtask-1: In one experiment, we failed to detect a significant amount of 1,25$(OH)_2$D by ELISA in the dLNs following subcutaneous injections of the different DCs. Potential reasons include: ***1)*** the ELISA method was not sufficiently sensitive; ***2)*** we had a problem extracting the 1,25$(OH)_2$D from the dLN tissues. We are working to improve the technique. Since one intravenous injection of the DC-CYP, as compared to no treatment and the wild type DC, showed significant suppression of the established experimental colitis, we have moved forward to the next tasks. 10% accomplished. 2. Subtask-2: As described above, these experiments were performed using intravenous injections of the different DCs. We currently have analyzed regulatory and gut-homing phenotypes of the T cells in the spleen and MLNs following one intravenous injection of each of the different DCs. Our data demonstrate that one intravenous injection of the DC-CYP, as compared to the no treatment and the wild type DCs, significantly increased the numbers of Treg cells that express foxp3, IL-10 and IL-4. In addition, one intravenous injection of the DC-CYP, as		

compared to the no treatment and the parental DCs, did not significantly enhance the expression of gut-homing receptor, CCR9 **(Figure 3)**. Additionally, we showed that a high 1,25$(OH)_2$D concentration was necessary to induce foxp3 expression *in vitro* (**Figure 1**). The data further underscores the importance of our engineered DC-CYP and DC-CYP-ALDH which can *de novo* synthesize a focally high concentration of 1,25$(OH)_2$D at the DC-T cell interface. 100% accomplished.		
Local IRB/IACUC Approval: April 30th 2015		
Milestone Achieved: HRPO/ACURO Approval: August 12th 2015		
Major Task 2: *to study regulatory function of the T cells in the dLNs in animals subcutaneously infused with the different DCs.*	4-12	Dr. Xiaolei Tang
Milestone(s) Achieved: As described above, these experiments were performed using intravenous injections of the different DCs. We currently have analyzed regulatory function of the $CD4^+$ T cells in the MLNs following two intravenous injections of each of the different DCs. Our data demonstrate that two intravenous injections of the DC-CYP, as compared to the no treatment and the parental DCs, significantly augments regulatory function of the $CD4^+$ T cells in the mesenteric lymph nodes **(Figure 4)**. 70% accomplished,		
Major Task 3: *to investigate gut-migration of the T cells in the dLNs in animals subcutaneously infused with the different DCs.*	7-15	Dr. Xiaolei Tang
Milestone(s) Achieved: As described above, these experiments were performed using subcutaneous injections of the different DCs. Our data demonstrate that two subcutaneous injections of the DC-CYP, as compared to the no treatment and the wild type DCs, did not significantly increased $Foxp3^+$ Treg number in the gut of healthy animals **(Figure 5)**. 70% accomplished.		
Major Task 4: *to investigate clinical symptoms and intestine histology in animals that initially show overt colitis and are subsequently infused subcutaneously with the different DCs.*	10-18	Dr. Xiaolei Tang
Milestone(s) Achieved: These experiments were performed using intravenous injections of the different DCs. Our data demonstrated that animals with established colitis receiving one intravenous injection of the DC-CYP, as compared to no immunization, had significantly increased survival rate and higher body weight (**Figure 6**). 100% accomplished.		
Specific Aim 2 *To study phenotypes, regulatory function, gut migration, and colitis suppression capability of the T cells in the draining lymph nodes in animals that receive no treatment, wild type DCs, or DCs carrying the over-expressed RALDH2.* We have successfully engineered DCs to overexpress the RALDH2 (DC-ALDH) **(Figure 1)**. During an initial pilot study, we found that the DC-ALDH, when injected once intravenously, did not suppress the established experimental colitis. Therefore, we did not further pursue this specific aim. 100% accomplished.		
Major Task 5: *to evaluate T cell phenotypes in the dLNs in animals subcutaneously infused with the different DCs.*	1-6	Dr. Xiaolei Tang
Subtask 1: *to determine RA concentration by ELISA in the dLNs following subcutaneous infusion of the different DCs.*	1-3	
Subtask 2: *to determine regulatory and gut-homing phenotypes*	1-6	

of the T cells in the dLNs following subcutaneous infusion of the different DCs.		
Milestone(s) Achieved:		
Major Task 6: *to study regulatory function of the T cells in the dLNs in animals subcutaneously infused with the different DCs.*	4-12	Dr. Xiaolei Tang
Milestone(s) Achieved:		
Major Task 7: *to investigate gut-migration of the T cells in the dLNs in animals subcutaneously infused with the different DCs.*	7-15	Dr. Xiaolei Tang
Milestone(s) Achieved:		
Major Task 8: *to investigate clinical symptoms and intestine histology in animals that initially show overt colitis and are subsequently infused subcutaneously with the different DCs.*	10-18	Dr. Xiaolei Tang
Milestone(s) Achieved:		
Specific Aim 3 *To study phenotypes, regulatory function, gut migration, and colitis suppression capability of the T cells in the draining lymph nodes in animals that receive no treatment, wild type DCs, DCs carrying the over-expressed 1α hydroxylase, DCs carrying the over-expressed RALDH2, or DCs carrying the two over-expressed enzymes.* We have successfully engineered DCs to overexpress the 1α-hydroxylase (DC-CYP), the RALDH2 (DC-ALDH), and both (DC-CYP-ALDH) **(Figure 1)**. During an initial pilot study, we found that the DC-ALDH, when injected once intravenously, did not suppress the established experimental colitis. Therefore, the DC-ALDH was excluded from further experiments.		
Major Task 9: *to evaluate T cell phenotypes in the dLNs in animals subcutaneously infused with the different DCs.*	1-6	Dr. Xiaolei Tang
Subtask 1: *to determine 1,25(OH)2D and RA concentrations by ELISA in the dLNs following subcutaneous infusion of the different DCs.*	1-3	
Subtask 2: *to determine regulatory and gut-homing phenotypes of the T cells in the dLNs following subcutaneous infusion of the different DCs.*	1-6	
Milestone(s) Achieved: 1. Subtask 1: In one experiment, we failed to detect a significant amount of 1,25$(OH)_2$D and RA by ELISA in the dLNs following subcutaneous injections of the different DCs. Potential reasons include: ***1)*** the ELISA method was not sufficiently sensitive; ***2)*** the sample preparation protocol did not work appropriately. We are working on improving the technique. Since one intravenous injection of each of the DC-CYP and the DC-CYP-ALDH, as compared to the controls, showed significant suppression of the established experimental colitis, we have moved forward to the next tasks. 10% accomplished. 3. Subtask 2: These experiments were performed using intravenous injections of the different DCs. We currently have analyzed regulatory and gut-homing phenotypes of the T cells in the spleen and MLNs following one intravenous injection of each of the different DCs. Our data demonstrate that one intravenous injection of the DC-CYP and DC-CYP-ALDH, as compared to other controls, significantly increased numbers of Treg cells that express foxp3, IL-10 and IL-4. In addition, one intravenous injection of the DC-CYP-ALDH, as compared to other controls, significantly enhances the expression of the gut-		

homing receptor, CCR9 on T cells **(Figure 3)**. Additionally, we showed that a high 1,25(OH)$_2$D concentration was necessary to induce foxp3 expression *in vitro* (**Figure 1**). The data further underscores the importance of our engineered DC-CYP and DC-CYP-ALDH which can *de novo* synthesize a focally high concentration of 1,25(OH)$_2$D at the DC-T cell interface. 100% accomplished.		
Major Task 10: *to study regulatory function of the T cells in the dLNs in animals subcutaneously infused with the different DCs.*	4-12	Dr. Xiaolei Tang
Milestone(s) Achieved: These experiments were performed using intravenous injections of the different DCs. We currently have analyzed regulatory function of CD4$^+$ T cells in the MLNs following two intravenous injections of each of the different DCs. Our data demonstrated that CD4$^+$ T cells from MLNs of the animals receiving two intravenous injections of either the DC-CYP or the DC-CYP-ALDH, as compared to other controls, significantly suppressed the induction of TNBS colitis **(Figure 4)**. 70% accomplished.		
Major Task 11: *to investigate gut-migration of the T cells in the dLNs in animals subcutaneously infused with the different DCs.*	7-15	Dr. Xiaolei Tang
Milestone(s) Achieved: These experiments were performed using subcutaneous injections of the different DCs. Our data demonstrated that two subcutaneous injections of the DC-CYP-ALDH, as compared to other controls, significantly increased number of foxp3$^+$ Treg cells in the gut of healthy animals **(Figure 5)**. 70% accomplished.		
Major Task 12: *to investigate clinical symptoms and intestine histology in animals that initially show overt colitis and are subsequently infused subcutaneously with the different DCs.*	10-18	Dr. Xiaolei Tang
Milestone(s) Achieved: These experiments were performed using intravenous injections of the different DCs. Our data demonstrated that animals with established colitis receiving one intravenous injection of the DC-CYP-ALDH, as compared to no immunization and the DC-CYP, had significantly increased survival rate and higher body weight (**Figure 6**). 100% accomplished.		

3.2 Accomplishments under these goals: All data figures are attached in appendix-1.

3.2a. A high concentration of the 1,25(OH)$_2$D is necessary for inducing the expression of foxp3 in T cells.

In *in vitro* cultures, 1,25(OH)$_2$D has been shown to augment the expression of foxp3 that is the master regulator of a group of Treg cells[14]. However, systemic *in vivo* supplementation of 1,25(OH)$_2$D appeared to at the most only transiently increase foxp3$^+$ Treg cells, let alone supplementation of 1,25(OH)$_2$D precursors (e.g. 25[OH]D)[22,25]. We reason that the failure in the *in vivo* induction of stable foxp3$^+$ Treg cells through systemic supplementation of 1,25(OH)$_2$D and its precursors may be due to an insufficient delivery of 1,25(OH)$_2$D to T cells that are being primed by dendritic cells (DCs). To address this potential, we first asked what is the concentration necessary for 1,25(OH)$_2$D to induce foxp3 expression in T cells that are being activated. Accordingly, we examined foxp3 expression in T cells during T cell activation in the presence of exogenous 1,25(OH)$_2$D at different concentrations. We found that, although a low 1,25(OH)$_2$D concentration (20nM) slightly up regulated foxp3 expression in the activated T cells, a high 1,25(OH)$_2$D concentration (100nM) was required to significantly augment foxp3 expression (**Fig 1**). In this regard, it has been shown that the normal range of serum

1,25$(OH)_2$D concentration in human is about 16 ~ 56 pg/ml (0.038nM ~ 0.13nM)[26,27]. Although some patients may tolerate up to 300pg/ml (0.72nM) of serum 1,25$(OH)_2$D level without the dose-limiting toxicity (i.e. hypercalcemia)[27], this dose (20nM) is far lower than the dose necessary for up regulating the foxp3 expression in our experimental system. Hence, we argue that this Treg-inducing 1,25$(OH)_2$D dose is difficult to reach through systemic supplementation of 1,25$(OH)_2$D and its precursors because of the dose-limiting toxicity[25]. Additionally, since 1,25$(OH)_2$D is a pleiotropic molecule[28], systemic supplementation of such a high dose may potentially lead to other unintended biological changes. Likewise, retinoic acid (RA) is also a pleiotropic molecule and therefore, systemic administration may cause undesired side effects[29].

3.2b. Bone-marrow-derived dendritic cells (BMDCs) can be engineered to *de novo* synthesize high concentrations of 1,25$(OH)_2$D and retinoic acid (RA).

Because of the anticipated difficulty in supplementing 1,25$(OH)_2$D and RA systemically for inducing gut-homing Treg cells, we propose that the Treg-inducing 1,25$(OH)_2$D concentration can be delivered by engineering a DC for the overexpression of the 1α-hydroxylase. To enhance the expression of gut-homing receptors in the newly induced Treg cells, we propose to further engineer the DC for the overexpression of RALDH2. We reason that the overexpressed 1α-hydroxylase in the DC produces a high concentration of 1,25$(OH)_2$D for the induction of foxp3 in T cells and the overexpressed RALDH2 produces a high concentration of RA to imprint gut-homing receptors in the same T cells. To investigate this potential, we first asked whether the BMDCs could be engineered to overexpress the 1α-hydroxylase and the RALDH2. To address this question, we generated three lentiviral vectors (**Fig 2A**): the lenti-CYP that carried the cytochrome P450 family 27 subfamily B member 1 (CYP27B1) gene for encoding the 1α-hydroxylase; the lenti-ALDH that carried the aldehyde dehydrogenase 1 family member A2 (ALDH1a2) gene for encoding the RALDH2; and the lenti-CYP-ALDH that carried both the CYP27B1 and the ALDH1a2 genes for encoding both the 1α-hydroxylase and the RALDH2 respectively.

Subsequently, the viral vectors were packaged into viral particles that were then used to transduce BMDCs. We showed that the cultured BMDCs displayed typical myeloid DC phenotype, i.e. protruding dendrites (**Fig 2B)**. In addition, more than 90% of the cultured BMDCs expressed CD11c (i.e. the marker for DCs) (**Fig 2C**). We then transduced the BMDCs with the lenti-CYP-ALDH (such transduced BMDCs are hereafter called BMDC-CYP-ALDH). Following the transduction, the BMDC-CYP-ALDH, as compared to the parental BMDCs, showed significantly enhanced expression of the 1α-hydroxylase as determined by flow cytometry (**Fig 2D**). To determine functional activity of the overexpressed 1α-hydroxylase, we added in the BMDC cultures 1α-hydroxylase substrate, i.e. the 25(OH)D (calcidiol) that is the major vitamin D form in the blood. The cells were further cultured for 24 hours and 1,25$(OH)_2$D concentration in the culture supernatants was examined. Our data showed that 1,25$(OH)_2$D concentration was significantly higher in the BMDCs that were transduced with the lenti-CYP (i.e. BMDC-CYP) and the BMDC-CYP-ALDH, as compared to the parental BMDCs and the BMDCs that were transduced with the lenti-ALDH (i.e. BMDC-ALDH) (**Fig 2E)**. Hence, our data demonstrated that the 1α-hydroxylase activity was significantly augmented in the BMDC-CYP and the BMDC-CYP-ALDH. To evaluate functional activity of the overexpressed RALDH2, we added a commercially produced non-toxic RALDH2 substrate, i.e. the BODIPY^{TM}-aminoacetaldehyde, into the BMDC cultures and measured production of the fluorescent product, i.e. the BODIPY^{TM}-aminoacetate by flow cytometry. Our data showed that the BMDC-CYP-ALDH, as compared to the parental BMDCs, displayed significantly enhanced RALDH2 activity (**Fig 2F**). To summarize, we have shown that the BMDC-CYP-ALDH, as compared to the parental BMDCs, secrete significantly higher concentrations of 1,25$(OH)_2$D and RA.

3.2c. DC-CYP-ALDH, as compared to other controls, induces more gut-homing Treg cells in spleen and MLNs.

Next, we investigated whether the DC-CYP and the DC-CYP-ALDH in the LPS (lipopolysaccharide)-activated form could induce Treg cells in the peripheral lymphoid tissues. We decided to use activated DC to examine whether our engineered DCs did not depend on a tolerogenic status because both tolerogenic DCs and adoptively transferred Tregs have been shown unstable in an *in vivo* pro-inflammatory environment[15,29-33]. In addition, in this experiment, we used a BMDC line, i.e. the DC2.4 that was a kind gift from Dr. Kenneth L. Rock[30]. It is important to mention that the DC2.4 cells have been shown to closely mimic the function of BMDCs[11,30,31]. Accordingly, the DC2.4 cells were engineered to overexpress the 1α-hydroxylase (DC2.4-CYP) or both the 1α-hydroxylase and the RALDH2 (DC2.4-CYP-ALDH). Subsequently, Balb/c mice received no immunization, 1 x 10^6 wild type DC2.4, 1 x 10^6 DC2.4-CYP, or 1 x 10^6 DC2.4-CYP-ALDH. Ten days later, spleens and mesenteric lymph nodes (MLNs) from the animals were examined for the expressions of markers for regulatory T cells (**Fig 3A**). Our data demonstrated that, as compared to the animals receiving the DC2.4, those receiving the DC2.4-CYP and DC2.4-CYP-ALDH displayed an increased number of foxp3$^+$ T cells in both spleen and MLNs (**Fig 3B and C**). Interestingly, a portion of the foxp3$^+$ T cells in the spleens but not the MLN co-expressed IL-17, suggesting that the engineered DC2.4 cells induced different Treg cells under different microenvironments. Furthermore, as compared to the DC2.4 and the DC2.4-CYP, the DC2.4-CYP-ALDH induced more foxp3$^+$ T cells. Finally, the DC2.4-CYP-ALDH but not the DC2.4 and the DC2.4-CYP consistently up regulated IL-4$^+$ and IL-10$^+$ T cells in both spleens and MLNs (**Fig 3D**). The above data suggest that the DC2.4-CYP-ADLH, as compared to the DC2.4-CYP, has a stronger ability to induce Treg cells.

With respect to the expressions of gut-homing receptors, we did not see significant difference in the expression of $\alpha4\beta7$ following the immunizations. However, it was consistent that the DC2.4-CYP-ALDH, but not other controls, significantly enhanced CCR9 expression in T cells in both spleens and MLNs (**Fig 3E and F**).

3.2d. Adoptive transfer of mesenteric lymph node (MLN) CD4$^+$ T cells from the animals intravenously immunized with either the BMDC-CYP or the BMDC-CYP-ADLH cells prevents the induction of experimental colitis.

Since most Treg cells have been defined in CD4$^+$ T cell subset, we proceeded to analyze the suppressive function of MLN CD4$^+$ T cells in the animals that received different immunizations. Accordingly, Balb/c mice were intravenously immunized with BMDC, BMDC-CYP, or BMDC-CYP-ALDH at days 0 and 10. Ten days after the second immunization, CD4$^+$ T cells in the MLNs were purified from the animals by a commercial CD4$^+$ T cell purification kit. Subsequently, 1 x 10^6 purified CD4$^+$ T cells were transferred into host Balb/c mice that were induced for colitis 3 days before the CD4$^+$ T cell transfer. In this experiment, a mild colitis was induced and all the animals spontaneously recovered after day 4. After a complete recovery, all the animals were induced for colitis for the second time for evaluating whether the transferred CD4$^+$ T cells could prevent the colitis induction (**Fig 4A**). Our data showed that transfer of the CD4$^+$ T cells from the animals immunized with both the BMDC-CYP and the BMDC-CYP-ALDH, but not the parental BMDCs, significantly suppressed the colitis induction (**Fig 4B**). Interestingly, the CD4$^+$ T cells from BMDC-CYP-immunized animals possessed similar suppressive activity as those from the BMDC-CYP-ALDH-immunized animals. In conclusion, the above data suggest that RA from the BMDC-CYP-ALDH did not enhance suppressive activity of the Treg cells induced by 1,25$(OH)_2$D.

3.2e. Foxp3$^+$ Treg cells, generated in the local draining lymph nodes by subcutaneous immunization with the BMDC-CYP-ALDH, migrate into intestines.

To determine whether antigen-specific Treg cells, induced in draining lymph nodes through subcutaneous immunization with the BMDC-CYP-ALDH, could migrate into intestines, Balb/c mice were subcutaneously immunized with $OVA_{323-339}$-pulsed BMDC, BMDC-CYP, or BMDC-CYP-ALDH cells at days 0 and 10. A group of mice that did not receive immunization were included as controls (No immu). Ten days after the second immunization, mononuclear cells in colon tissues from all the mice were analyzed by flow cytometry for the $OVA_{323-339}$-specific T cells, identified by $OVA_{323-339}$/I-A^d tetramer, among $foxp3^+$ T cells (**Fig 5A**). Our data showed that, following subcutaneous immunization with the BMDC-CYP-ALDH, but not the BMDC and the BMDC-CYP, the $OVA_{323-339}$/I-A^d $tetramer^+$ cells among $foxp3^+$ T cells were significantly increased in the colon tissues (**Fig 5B, C, and D**). The data therefore suggest that the gut-homing $OVA_{323-339}$-specific, $foxp3^+$ Treg cells were induced in the local draining lymph nodes and successfully homed to the colon tissues.

3.2f. Intravenous immunization with the BMDC-CYP-ALDH arrests the progression of and promotes the recover from established experimental colitis.

Since we have demonstrated that T cells induced by the BMDC-CYP-ALDH possess regulatory property and migrate into colons, we reason that the BMDC-CYP-ALDH may have a potential to treat IBD. To address this question, animals were induced for severe experimental colitis. At day 3, the animals received one of the following immunizations: ***1)*** no immunization (No immu); ***2)*** the BMDC-CYP; ***3)*** the BMDC-CYP-ALDH. Additionally, a group of healthy animals were also included as controls (**Fig 6A**). We found that, although the animals induced for the colitis without any immunization displayed 100% mortality, the animals immunized with the BMDC-CYP-ALDH showed 0% mortality and the animals immunized with the BMDC-CYP had about 50% mortality (**Fig 6B, upper panel**). In addition, the animals immunized with the BMDC-CYP-ALDH, as compared to those with the BMDC-CYP, were much quicker in gaining body weight (**Fig 6B, lower panel**). Furthermore, colons from the animals were examined at day 14. Our data showed that animals induced for the colitis without any immunization, as compared to those with the BMDC-CYP and the BMDC-CYP-ALDH, displayed significantly shortened colon length. Additionally, colon length in animals immunized with the BMDC-CYP-ALDH, as compared to the BMDC-CYP, was significantly longer. Indeed, the colon length in animals immunized with the BMDC-CYP-ALDH was similar to that in the healthy control animals (**Fig 6C**). Finally, H&E staining showed that colon tissues from animals immunized with the BMDC-CYP-ALDH, as compared to those without any immunization, were less damaged (**Fig 6D**).

3.3 **Opportunities for training and professional development:** Nothing to Report.

3.4 **Results disseminated to communities of interest?**

3.4a. Poster presentation at International Congress of Immunology 2016, August 21 to 26, 2016. Melbourne, Australia. (Abstract attached in Appendix-2). This meeting was supported by an American Association of Immunologists Travel Award to Xiaolei Tang.

3.5 **Future plan to accomplish the goals:** During the next report period, we will further perform the following studies:

3.5a. Investigate whether intravenous and/or subcutaneous delivery of the DC-CYP-ALDH, as compared to the DC and the DC-CYP, provides a much stronger suppression of the established experimental colitis.

3.5b. Further improve the techniques for evaluating concentrations of 1,25(OH)2D and RA in the peripheral lymphoid tissues, e.g. draining lymph nodes and spleen.

3.5c. Continue to investigate regulatory function of the gut-homing Treg cells induced by the engineered DCs.

3.5d. Continue to study migration of the antigen-specific, gut-homing Treg cells induced by the engineered DCs to the gut.

4. IMPACT:

4.1 Impact on the development of the principle discipline of this project: Currently, vitamin D and its metabolites have been evaluated for potential treatment of IBD through systemic supplementation. However, these clinical studies have not produced the expected outcome. This hurdle may be due to the inability to generate a sufficient $1,25(OH)_2D$ concentration in the peripheral lymphoid tissues to execute the $1,25(OH)_2D$'s immune regulatory function and at the same time to spare the severe systemic side effects (e.g. hypercalcemia) that are potentially associated with a high $1,25(OH)_2D$ concentration in the blood. Our results have clearly shown that gut-homing Treg cells can be successfully induced in the peripheral lymphoid tissues by the DCs engineered to overexpress the 1α-hydroxylase and RALDH2. Additionally, such induced Treg cells can successfully migrate into the gut. Therefore, our data could definitively address the therapeutic potential of $1,25(OH)_2D$ as an immune regulator for IBD.

4.2. Impact on other disciplines: Since attempts to utilize $1,25(OH)_2D$'s immune regulatory function to treat other diseases including other autoimmune diseases and cancers are not satisfactory. These unsatisfactory results may be due to the same hurdle being addressed in this project. Therefore, our results may also help resolve $1,25(OH)_2D$'s therapeutic role in other diseases as well.

4.3 Impact on technology transfer: Based on our promising results, we have filed a PCT patent application (international application No# PCT/US2016/017610).

4.4 Impact on society beyond science and technology: Nothing to Report.

5. CHANGES/PROBLEMS:

***5.1 Changes in approach and reasons for change*:** During an initial pilot study, we found that the DC-CYP, when injected once intravenously as compared to subcutaneously, provided much stronger suppression of established experimental colitis. Therefore, except for the determination of $1,25(OH)_2D$ concentration in the draining lymph nodes and the gut-migration of newly induced Treg cells in which the different DCs were injected subcutaneously, other experiments were performed using intravenous injection of each of the different DCs. Since subcutaneous injection is more convenient for IBD patients, we will continue to evaluate whether multiple subcutaneous injections can reach the therapeutic effect of intravenous injection.

5.2 Actual or anticipated problems or delays and actions or plans to resolve them:

5.2a In a pilot study, we found that the DC-ALDH, when injected once intravenously, did not suppress established experimental colitis. Therefore, we did not further pursue the specific aim 2.

5.2b In one experiment, we failed to detect the $1,25(OH)_2D$ and RA concentrations in the local draining lymph nodes following subcutaneous injection of the DC-CYP-ALDH. We are considering the following reasons: ***1)*** unsuccessful extraction of $1,25(OH)_2D$ from draining lymph nodes; ***2)*** inappropriate sample preparation; ***3)*** the ELISA methods are not sensitive enough. To resolve the above issues, we will send the samples to a company for detecting $1,25(OH)_2D$ using more sensitive methods, e.g. radioimmunoassay. In addition, we will continue to improve the techniques for preparing the samples for measuring $1,25(OH)_2D$ and RA.

6. PRODUCTS:

***6.1 Conference paper*:** Poster presentation at International Congress of Immunology 2016, August 21 to 26, Melbourne, Australia 2016. (Abstract attached in Appendix-2). This meeting was supported by an American Association of Immunologists Travel Award to Xiaolei Tang.

6.2 Patent application: we have filed a PCT patent application (international application No# PCT/US2016/017610). The patent information is attached in Appendix-3.

6.3 Manuscript in preparation: Yi Xu*, Yanmei Cheng*, David J. Baylink, Mei Huang, Christian Chan, Hannah Chelliah, Chih-Huang Li, Xiaohua Wang, Jintao Zhang, William Lau, , Xuezhong Qin, Xiaolei Tang. Dendritic Cells Engineered to *De Novo* Synthesize High Concentrations of Both the Calcitriol and Retinoic Acid Prime Gut-homing Regulatory T cells and Rapidly Arrest the Progression of Established Experimental Colitis. In preparation.

7. PARTICIPATNS & OTHER COLLABORATING ORGANIZATIONS:

7.1 Individuals who worked on the project:

Name	Project Role	Position	Percent	Funding support
Xiaolei Tang	Principle Investigator	Assistant Professor	25%	
Xuezhong Qin	Co-investigator	Associate Professor	2%	Internal
Yi Xu	Daily research	Postdoctoral fellow	50%	50% internal funding
Edmundo Carreon Berumen	Daily laboratory support	Technician	20%	Internal funding

7.2 Changes in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period: Dr. Xiaolei Tang has a new active internal Innovation Grant support shown below:

681207-2967 (Tang & Goel) 02/01/16 ~ 01/31/18
Loma Linda University, Department of Medicine, GI Foundation.
"Suppression of Experimental Colitis through Enhancing Mucosal Repair and Reinstating Immune Tolerance in the Inflamed Gut"
The major goals of this project are to develop a novel combination therapy which accelerates repair of disrupted mucosal barrier as well as specifically strengthens immune regualtion in the intestines.
Role: co-PI.

7.3 Other organizations involved as partners: Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS: N/A.

9. APPENDICES:

9.1 Appendix-1: Figures
9.2 Appendix-2: Abstract presented at the ICI 2016.
9.3 Appendix-3: Patent application: PCT/US2016/017610.
9.4 Appendix-4: References cited.

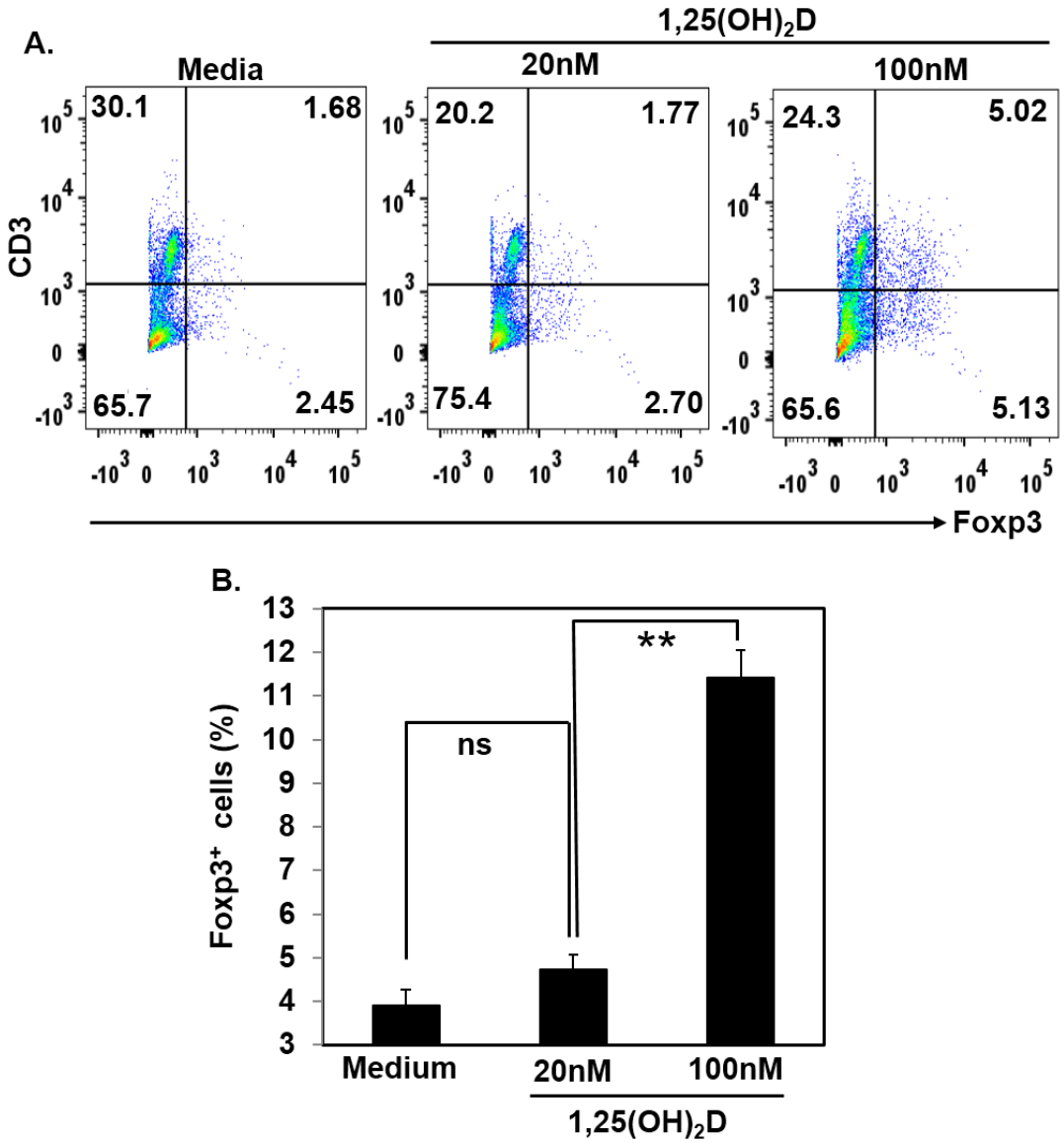


Figure 1. A high concentration of the 1,25(OH)$_2$D is necessary for inducing the expression of foxp3 in T cells. Splenocytes were co-cultured with the anti-CD3/CD28 magnetic beads in the presence or absence of 1,25(OH)$_2$D at a low (20nM) or high (100nM) concentration. Seventy-two hours later, the cells were analyzed for the expression of foxp3 in T cells (CD3$^+$) by flow cytometry. ***A)*** Representative FASC plots show expression of the foxp3 in T cells in the indicated cultures. ***B)*** Cumulative data from "A". Data are means ± SEM (n=4-6) and are representative of two independent experiments. ** $P < 0.01$. ANOVA test.

APPENDIX-1: Figures

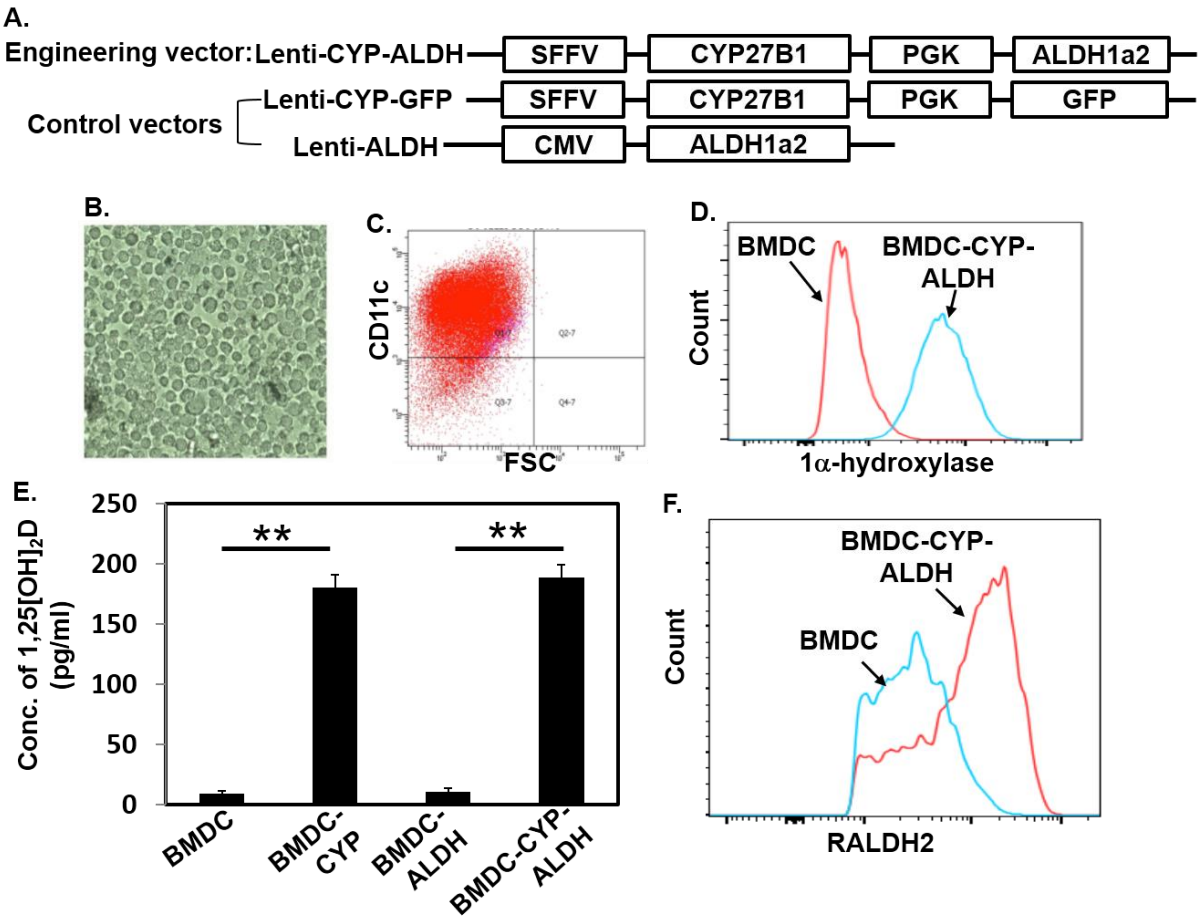


Figure 2. Bone-marrow-derived dendritic cells (BMDCs) can be engineered to de novo synthesize high concentrations of both 1,25(OH)$_2$D and retinoic acid. Dendritic cells (DC) were generated from bone marrow and transduced with the indicated lenti-viral vectors as described in the "Materials and Methods". ***A)*** Lenti-viral vectors used in this study. Lenti-CYP-ALDH: the lenti-viral vector that expressed both the CYP27B1 and the ALDH1a2 genes. Lenti-CYP-GFP: the lenti-viral vector that expressed both the CYP27B1 and the GFP genes. Lenti-ALDH: the lenti-viral vector that expressed the ALDH1a2 gene. "SFFV", "PGK", and "CMV" are promoters. ***B)*** A representative microscope image of the cultured BMDCs. ***C)*** A representative FACS plot shows expression of the parental BMDC marker, i.e. the CD11c. ***D)*** A representative FASC plot shows the expression of 1α-hydroxylase in the parental BMDCs and the 1α-hydroxylase-RALDH2-overexpressing BMDCs (BMDC-CYP-ALDH). ***E)*** 1α-hydroxylase substrate (25[OH]D) was added into the cultured BMDCs. After overnight incubation, supernatants were collected and measured for production of the active vitamin D metabolite, i.e. 1,25(OH)$_2$D. The data show concentration of 1,25(OH)$_2$D in the parental BMDCs, the 1α-hydroxylase-overexpressing BMDCs (BMDC-CYP), the RALDH2-overexpressing BMDCs (BMDC-ALDH), and the BMDC-CYP-ALDH. **P<0.01. ***F)*** RALDH2 activity was measured using the ALDEFLUOR assay. A representative FACS plot shows the RALDH2 activity in the parental BMDCs and the BMDC-CYP-ALDH.

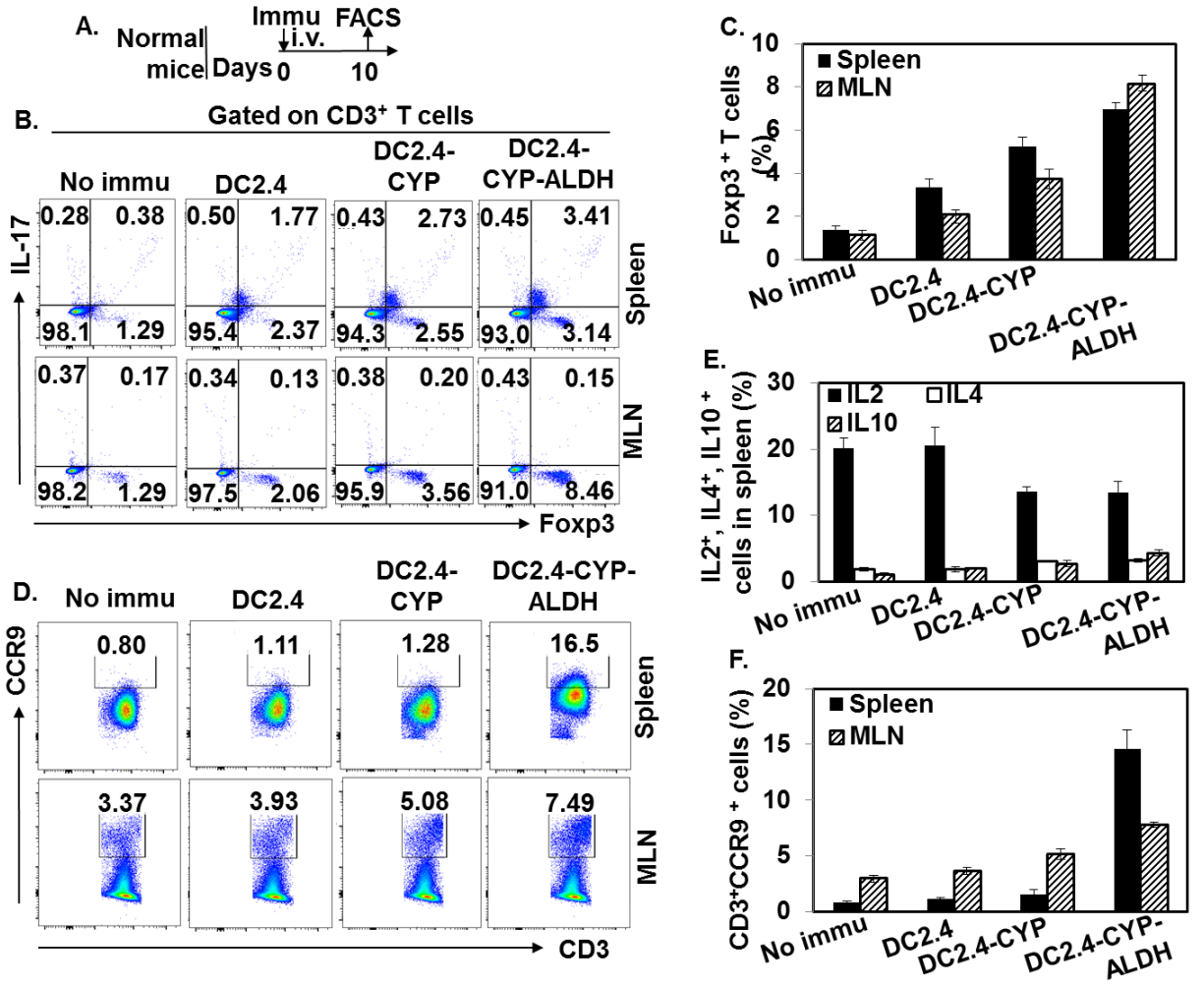


Fig 3. DC-CYP-ALDH, as compared to other controls, induces more gut-homing Treg cells in spleen and MNLs. DC2.4 cell, which is a BMDC line and closely mimics primary BMDC function[1-3], was engineered to overexpress the 1α-hydroxylase (DC-CYP) or both the 1α-hydroxylase and the RALDH2 (DC-CYP-ALDH). Healthy Balb/c mice received no treatment, 1 x 10^6 DC2.4, 1 x 10^6 DC-CYP, or 1 x 10^6 DC-CYP-ALDH. Ten days later, spleens and mesenteric lymph nodes (MLNs) from the animals were analyzed for expressions of IL-17, foxp3, CCR9, $\alpha4\beta7$, IL-2, IL-10, and IL-4 in T cells. ***A)*** Experimental design. ***B)*** Representative FACS plots showing expression of foxp3 and IL-17 in spleens and MLNs. ***C)*** Cumulative data from the experiment in "B". Spleen: DC v.s DC-CYP P<0.05; DC vs DC-CYP-ALDH P<0.01; DC-CYP v.s DC-CYP-ALDH P<0.01. MLN: DC v.s DC-CYP P<0.05; DC vs DC-CYP-ALDH P<0.01; DC-CYP v.s DC-CYP-ALDH P<0.01. ***D)*** Representative FACS plots showing expression of CD3 and CCR9. ***E)*** Cumulative data showing percent of $CD3^+CCR9^+$ T cells in spleens and MLN from the experiment in "D". Spleen: DC v.s DC-CYP P=0.11; DC vs DC-CYP-ALDH P<0.01; DC-CYP v.s DC-CYP-ALDH P<0.01. MLN: DC v.s DC-CYP P<0.01; DC vs DC-CYP-ALDH P<0.05; DC-CYP v.s DC-CYP-ALDH P<0.01. ***F)*** Cumulative data showing percent of $IL\text{-}2^+$, $IL\text{-}4^+$, and $IL\text{-}10^+$ cells in the spleens and MLN from the experiment in "D". IL-2: DC v.s DC-CYP P<0.05; DC vs DC-CYP-ALDH P<0.05; DC-CYP v.s DC-CYP-ALDH P=0.45. IL-4: DC v.s DC-CYP P<0.05; DC vs DC-CYP-ALDH P<0.01; DC-CYP v.s DC-CYP-ALDH P=0.27. IL-10: DC v.s DC-CYP P=0.09; DC vs DC-CYP-ALDH P<0.05; DC-CYP v.s DC-CYP-ALDH P<0.01.

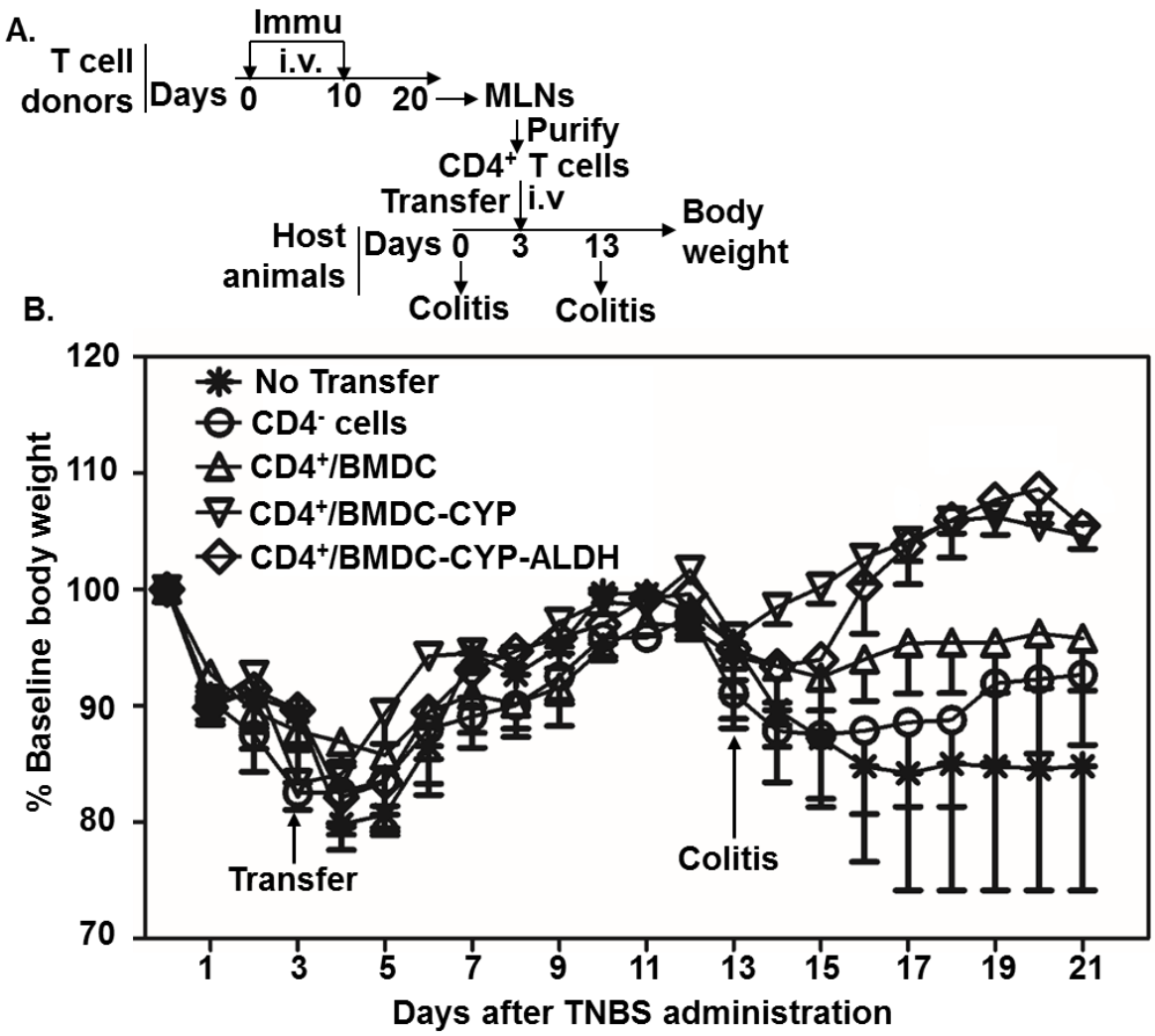


Fig 4. Adoptive transfer of $CD4^+$ T cells in the mesenteric lymph nodes (MLN-CD4) from animals intravenously treated with the DC-CYP and the DC-CYP-ADLH cells, as compared to other controls, prevent induction of experimental colitis. Balb/c mice were immunized with the DC, DC-CYP, or DC-CYP-ALDH at days 0 and 10. Ten days after the second immunization, 1 x 10^6 purified MLN-CD4 were intravenously transferred into host Balb/c mice that were induced for the experimental colitis 3 days before the cell transfer. On day 14, the animals were induced for the experimental colitis for the second time and monitored for body weight on a daily basis. ***A)*** Experimental design. ***B)*** Data show percent of baseline body weight with time. Data are presented as means ± SEM (n=4-6) and are representative of two independent experiments. DC v.s DC-CYP $P<0.01$; DC vs DC-CYP-ALDH $P<0.01$; DC-CYP v.s DC-CYP-ALDH $P>0.05$.

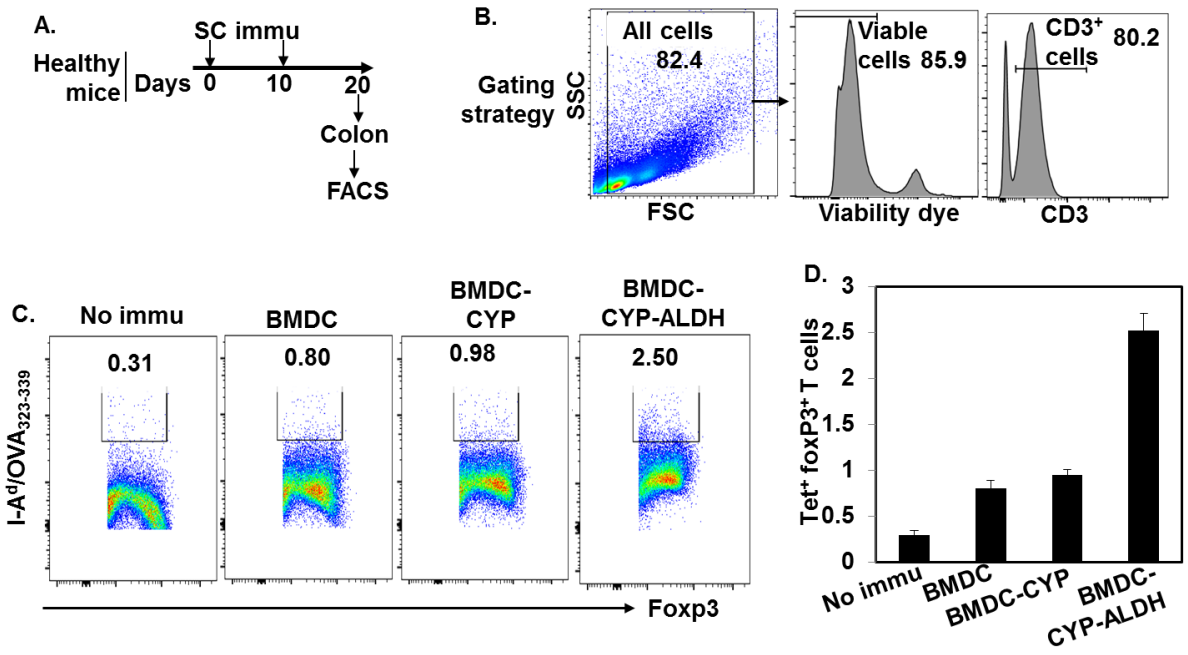


Fig 5. Foxp3$^+$ Treg cells, generated in the local draining lymph nodes by subcutaneous injection of the DC-CYP-ALDH, migrate into intestines. Balb/c mice were subcutaneously immunized with $OVA_{323-339}$-pulsed DC, DC-CYP, or DC-CYP-ALDH cells at days 0 and 10. A group of mice that did not receive any treatment were included as controls (No Tx). Ten days after the second immunization, colon tissues from all the mice were analyzed for $OVA_{323-339}$-specific T cells, identified by $OVA_{323-339}$/I-A^d tetramer, among foxp3$^+$ T cells. ***A)*** Experimental design. ***B)*** Gating strategy. ***C)*** Representative FACS plots showing $OVA_{323-339}$/I-A^d tetramer$^+$ cells among foxp3$^+$ T cells. ***D)*** Cumulative Data are presented as means ± SEM (n=4-6) and are representative of two independent experiments. DC v.s DC-CYP P=0.13; DC vs DC-CYP-ALDH P<0.01; DC-CYP v.s DC-CYP-ALDH P<0.01.

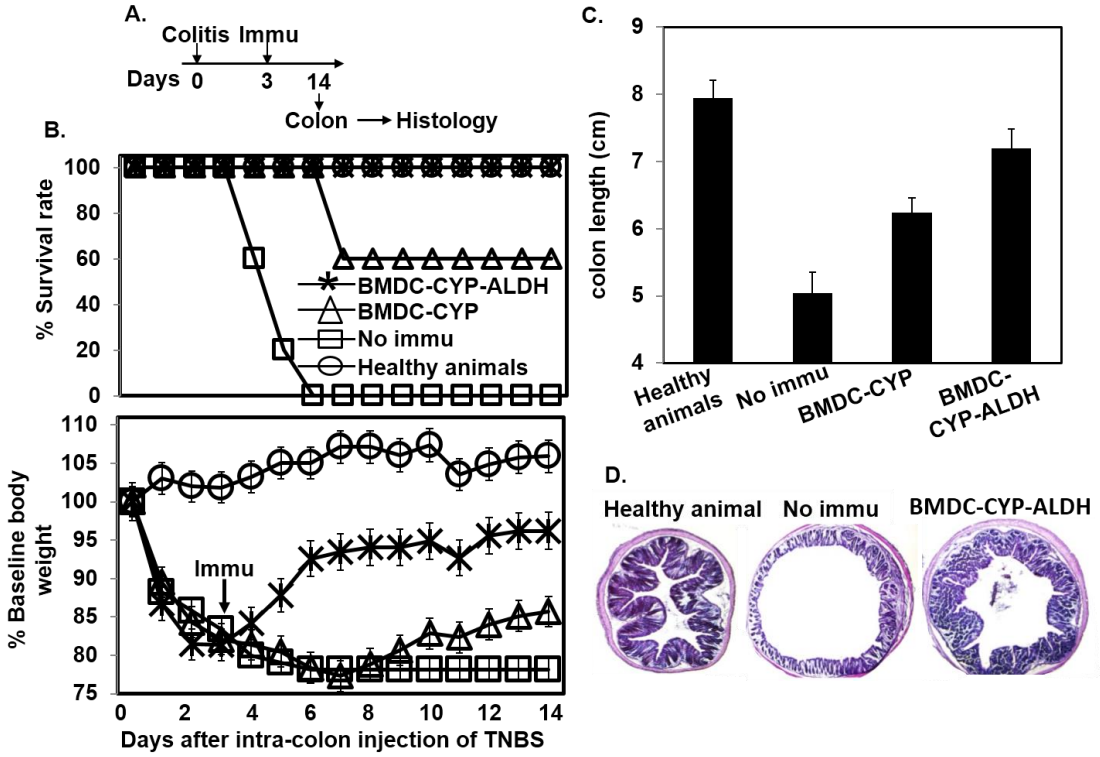


Figure 6. Intravenous immunization with the 1α-hydroxylase-RALDH2-overexpressing bone-marrow-derived DCs (BMDC-CYP-ALDH) arrests the progression of and promotes the recover from established experimental colitis. Balb/c mice were induced for TNBS colitis. At day 3, mice intravenously received one of the following immunizations (immu): ***1)*** no immunization (No immu); ***2)*** the 1α-hydroxylase-overexpressing BMDCs (BMDC-CYP); ***3)*** the BMDC-CYP-ALDH. Additionally, a group of healthy animals was included as a control. ***A)*** Experimental design. ***B)*** Survival (upper panel) and percent of baseline body weight (lower panel) are shown over the observation period. BMDC-CYP v.s No immu: $P<0.01$; BMDC-CYP-ALDH v.s No immu: $P<0.01$; BMDC-CYP-ALDH v.s BMDC-CYP: $P<0.05$. Data are means ± SEM (n=4-6) and are representative of two independent experiments. ***C)*** A comparison of colon lengths among the experimental animals. BMDC-CYP v.s No immu: $P<0.01$; BMDC-CYP-ALDH v.s No immu: $P<0.01$; BMDC-CYP-ALDH v.s BMDC-CYP: $P<0.05$. Data are Means ± SEM (n=4-6) and are representative of two independent experiments. ***D)*** Representative images of the H&E stained colons from the experimental animals.

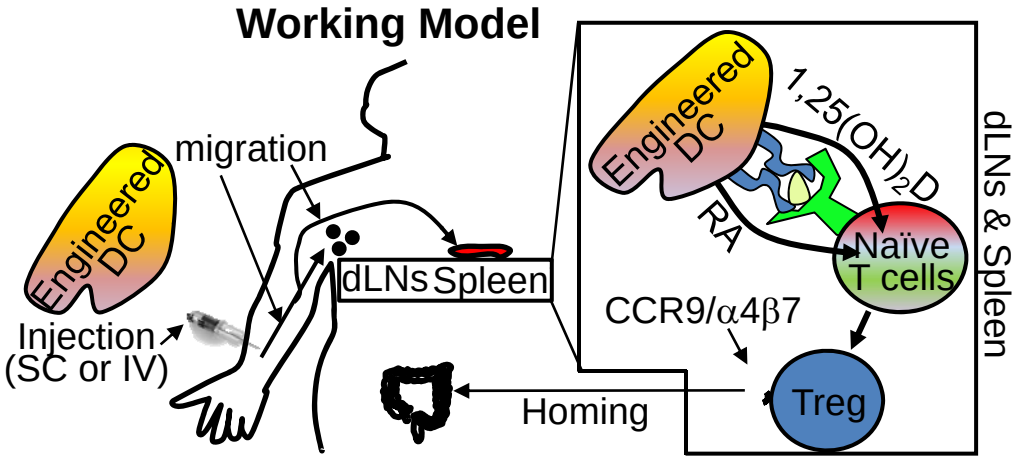
Dendritic Cells Engineered for *De Novo* Synthesis of Calcitriol and Retinoic Acid Prime Gut-homing Regulatory T Cells and Rapidly Arrest Progression of Ongoing Experimental Colitis

Yi Xu*, Yanmei Cheng*, Christian Chan, Hannah Chelliah, Chih-Huang Li, Xiaohua, Wang, Xuezhong Qin, William Lau, David J. Baylink, Xiaolei Tang. Loma Linda University, Loma Linda, CA USA

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder in the gastrointestinal tract (GUT) and does not have a permanent cure. Recent immunological interventions, e.g. TNF-α and $\alpha4\beta7$ blockers, have improved specificity in the control of gut inflammation and slowed disease progression. However, off target immune suppression is still causing unbearable adverse side effects, e.g. infections and cancers. Since deficiency of immune regulation in the intestines is a major mechanism underlying IBD, this study aims to induce gut-homing regulatory T (Treg) cells in the peripheral lymphoid tissues such that Treg cells in the intestines of IBD patients are selectively augmented to further increase specificity of immunotherapy. Here we showed that enhanced generation of gut-homing Treg cells was achieved through immunization with dendritic cells engineered to *de novo* synthesize a high concentrations of both the active vitamin D metabolite and the retinoic acid (RA). In addition, immunization with such engineered DCs rapidly arrested progression of experimental colitis induced by 2,4,6-trinitrobenzene sulfonic acid (TNBS).

Working Model



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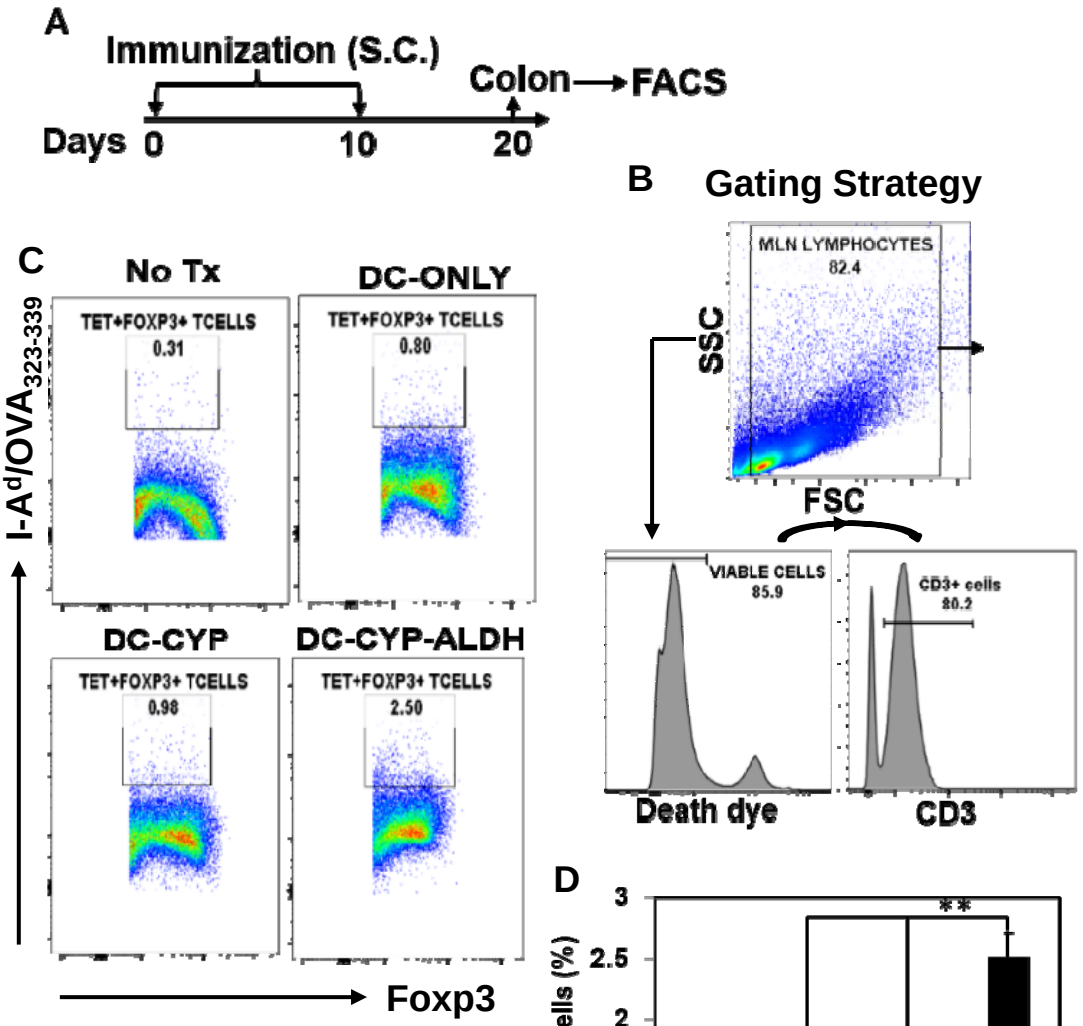

Figure 1. Immunization with dendritic cells engineered to *de novo* synthesize high concentrations of both calcitriol and retinoic acid (DC-CYP-ALDH) primes gut-homing regulatory T cells.

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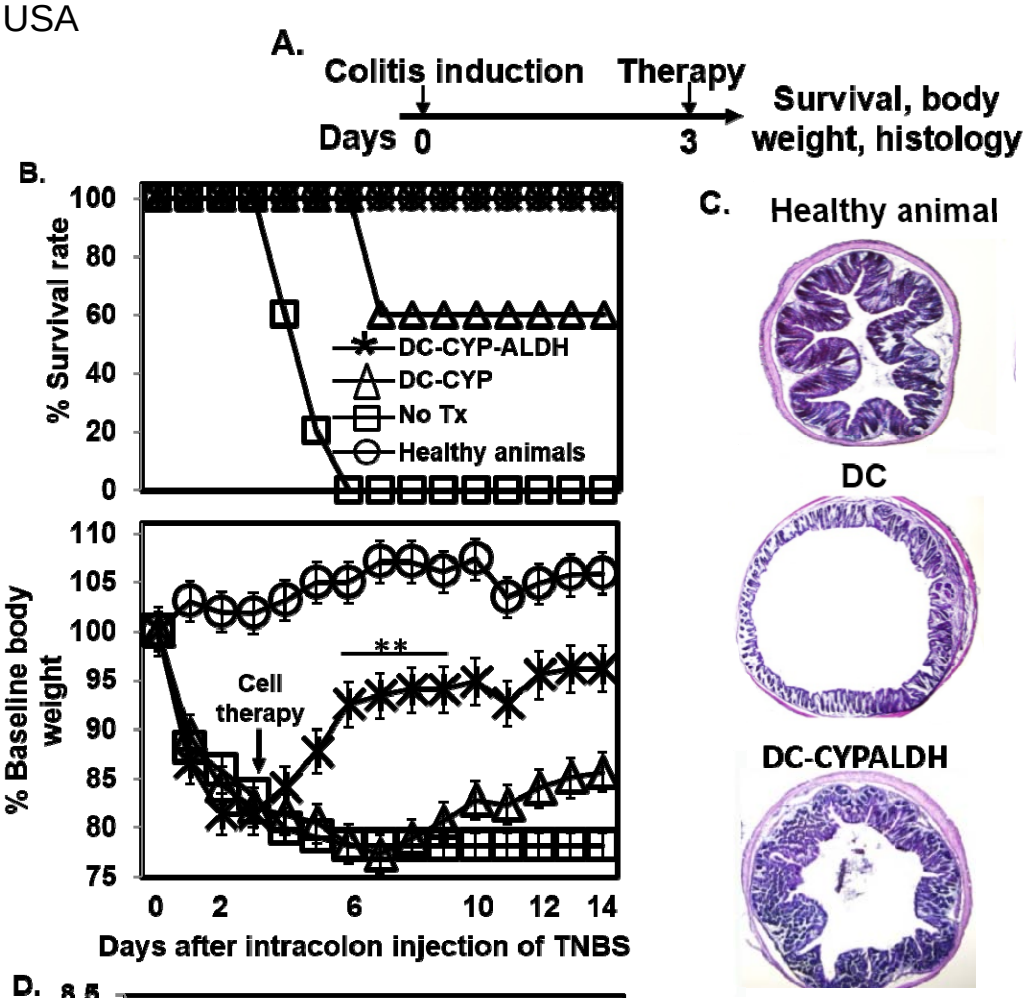

Figure 2. Immunization with dendritic cells engineered to *de novo* synthesize high concentrations of both calcitriol and retinoic acid (DC-CYP-ALDH) suppresses ongoing experimental colitis.

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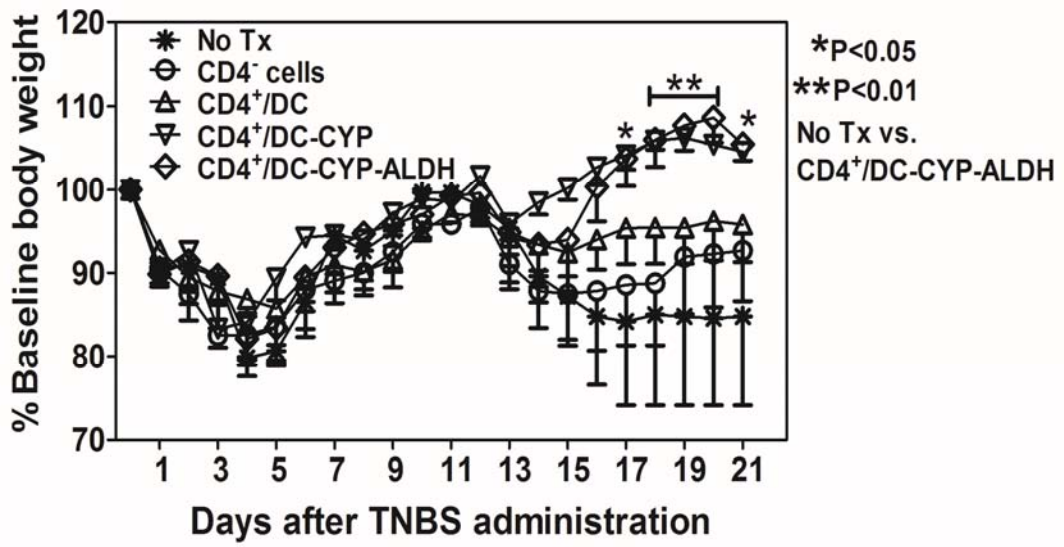

Figure 3. DC-CYP-ALDH immune $CD4^+$ T cells transfer suppression of experimental colitis.

Conclusion

Immunization with dendritic cells engineered to *de novo* synthesize high concentrations of calcitriol and retinoic acid primes gut-homing Treg cells, suppresses ongoing experimental colitis, and thereby is a promising gut-specific therapy for inflammatory bowel disease.

Acknowledgement

1. This work was supported by the Office of the Assistant Secretary of Defense for Health Affairs, through Peer Reviewed Medical Research Program under the award number W81XWH-15-1-0240.

2. This work was also partially supported by the Department of Medicine at Loma Linda University through the Research Innovation Grant - Gastroenterology, Hepatology and Nutrition (RIG-GI) under the award number 681207.

PATENT COOPERATION TREATY PCT/US2016/017610

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Date of mailing *(day/month/year)* 04 March 2016 (04.03.2016)	
Applicant's or agent's file reference LOMARM-002WO	**IMPORTANT NOTIFICATION**
International application No. PCT/US2016/017610	International filing date *(day/month/year)* 11 February 2016 (11.02.2016)
International publication date *(day/month/year)* **Not yet published**	Priority date *(day/month/year)* 11 February 2015 (11.02.2015)
Applicant LOMA LINDA UNIVERSITY	

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